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13. ABSTRACT (Maximum 200 Words)

This study is a feasibility study of a novel immunotherapeutic strategy for the treatment of breast cancer. The rationale is based upon recent findings that genes belonging to the pp32 family are differentially and alternatively expressed in most human breast cancers. In general, benign breast tissues express pp32, a tumor suppressor, whereas breast cancers express tumorigenic family members, including pp32r1 and pp32r2. Since pp32r1 and pp32r2 are expressed in nearly all breast cancers, but not in normal adult tissues, they may reasonable serve as targets for antigen-specific immunotherapy. The purpose of this study is to identify tumor-associated antigens (TAA) in pp32r1 and pp32r2, then test their suitability in vitro as immunotherapeutic targets in breast cancer. Currently, the animal study is underway. If successful, the results may translate into eventual clinical trials of peptide vaccines or adoptive T cell therapy.

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Introduction:

In the IDEA proposal, we proposed a feasibility study of a novel immunotherapeutic strategy for the treatment of breast cancer. The rationale is based upon recent findings that genes belonging to the pp32 family (Figure 1) are differentially and alternatively expressed in most human breast cancers. In general, benign breast tissues express pp32, a tumor suppressor, whereas breast cancers express tumorigenic family members, including pp32r1 and pp32r2. Since pp32r1 and pp32r2 are expressed in nearly all breast cancers, but not in normal adult tissues, they may reasonably serve as targets for antigen-specific immunotherapy.

Body:

Statement of Works:

Task 1. Identify, synthesize and test candidate peptides that could potentially bind to HLA class I molecules based on the coding sequence of pp32r1 and pp32r2. (Month 1-6)

Task 2. Screen *in vitro* for candidate pp32r1 & pp32r2 peptides that fulfill the requirements for TAA. (Month 7-12)

Task 3. Evaluate the pp32r1/pp32r2- specific cytotoxicity against a broad range of natural targets (established or primary breast cancer cell lines) to determine range of applicability.(Month 13-20)

Task 4. Evaluate *in vivo* immunogenicity of pp32r1 and/or pp32r2-derived TAAs in human breast cancer animal models. (Month 21-36)

In the first year of this project, we successfully identified two candidate TAA epitopes, which are capable of triggering MHC Class I dependent CTL response in vitro against artificial target cells. In the second year of this project, we further evaluated the applicability of the above candidate TAA epitopes against nature target cells (Task #3). In addition, we currently proceed into the early stage of *in vivo* study (Task #4)

1) Task #1: Identify, synthesize and test candidate peptides that could potentially bind to HLA class I molecules based on the coding sequence of pp32r1 and pp32r2.

Using Bioinformatics and ImmunoGenetics tools, we analyzed the entire coding region of pp32, pp32r1 and pp32r2 genes for binding affinity with HLA-A*0201 molecule as well as the degradation pattern by proteasomal cleavages. The result of calculation shown (Table 1) that 19 motifs are potentially favorable of binding to HLA-A*0201 molecule with high affinity. To verify the prediction *in vitro*, HLA-A*0201+ TAP-deficient T2 hybridoma (ATCC) was pulsed with 50ug/ml of each peptide representing the motif (or control) and 5ug/ml of b2-microglobulin for 18hr at 37 C. HLA-A*0201 expression was then measured by flow cytometry using mAb BB7.2 (ATCC) followed by incubation with FITC-conjugated secondary antibody. Fluorescent index of HLA-A*0201 to each peptide can be determined as: (mean fluorescence with peptide - mean fluorescence without peptide) / (mean fluorescence without peptide). The result shown 10 out of 20 motifs is capable of binding to HLA-A*0201 in a concentration dependent manner (Table 1).

2) Task #2: Screen for candidate pp32r1 & pp32r2 peptides that fulfill the requirements for TAA. In order to be qualified as a TAA, a motif has to be able to meet several criteria in addition to the binding to HLA-A*0201. These requirements include (i) the antigen can be naturally processed by tumor cells, (ii) it permits expansion of antigen-specific CTL; (iii) it is presented in a MHC-restricted fashion. CTL assay was carried out to test if the motifs identified in Aim#1 fulfill the requirements for TAA.

In brief, Cr^{51}-labeled target cells (T2 cells pulsed with peptide or cancer cell expressing pp32 family members) were incubated with various numbers of CTL effecter cells for 4 hr. Cr^{51}-release assays were performed in triplicate per condition using $5x10^3$ labeled target cells per well in a 96-well plate. Percent specific lysis will be calculated from CPM of (experimental result - spontaneous release)/(maximum release - spontaneous release). The results, summarized in Table 2, indicate that 2 out of 10 motifs fulfilled the above requirement as TAA.

3) Task #3. Evaluate the applicability of the pp32r1/pp32r2- specific cytotoxicity against a broad range of natural targets.

To evaluate the applicability of the pp32r1/pp32r2- specific cytotoxicity against a broad range of natural targets, primary cultures of breast tumor that are both HLA-A*0201 positive and pp32r1 / pp32r2 positive was selected as target cells. The expression of HLA-A*0201 was verified by flow cytometry, whereas the expression of pp32r1 and/or pp32r2 was confirmed by subtype-specific RT-PCR. CTL assay was carried out to test if the motifs identified in Task #1&#2 are applicable to HLA-A*0201 positive and pp32r1 / pp32r2 positive primary cultures. In brief, Cr^{51}-labeled target cells were incubated with various numbers of CTL effecter cells for 4 hr. Cr^{51}-release assays were performed in triplicate per condition using $5x10^3$ labeled target cells per well in a 96-well plate. Percent specific lysis will be calculated from CPM of (experimental result - spontaneous release)/(maximum release - spontaneous release). Unlike the artificial target cells used in Aim#2, the results shown no detectible pp32r1/pp32r2- specific cytotoxicity against primary cultures of breast tumor that are both HLA-A*0201 positive and pp32r1 / pp32r2 positive. A possible explanation might be the difference in expression/presentation of pp32r1 / pp32r2 between primary cells and artificial target cells.

Due to the high homology among pp32 family members (over 90% identity at amino acid level), none of the existing antibodies is subtype- specific. Therefore, the reliable method to screen pp32r1 and pp32r2 expression has been based on RT-PCR. Although this screen method is very effective to identify cells/tissue that express pp32r1 and pp32r2 at mRNA level, its result may not correlate with the expression of pp32r1 and pp32r2 at the protein level, which is crucial for evaluating pp32r1/pp32r2- specific cytotoxicity. As an alternative, current efforts are being made to establish subtype- specific antibodies so that a reliable method to test the expression of pp32r1 / pp32r2 at protein level will be available to re-evaluate the applicability of the pp32r1/pp32r2- specific cytotoxicity against natural target cells.

4) Specific Aim 4. Evaluate *in vivo* immunogenicity of pp32r1 and/or pp32r2-derived TAAs in human breast cancer animal models. This phase of study includes (i) evaluate whether the identified TAAs are capable of triggering the expansion of pp32r1/ pp32r2-specific CTL and antigen-specific CTL response *in vivo*, (ii) Study anti-tumor activity of pp32r1/pp32r2- specific CTLs in breast cancer xenograft model. We are currently in the process of testing and validifying animal models.

Key Research Accomplishments:

We have identified two peptide motifs from pp32 family members, which fulfill the requirement to be TAAs. This study provided bases for further feasibility study of pp32r1 and pp32r2 as target breast cancer immunotherapy.

Reportable Outcomes:

The result of Specific Aim #1 and #2 were presented at 2002 Era of Hope Department of Defense Breast Cancer Research Program Meeting.
Yu, W., Jagun, A., Zhu, X., Jaffee, EM, & Bai, J. Identification of Candidate Tumor-Associated Antigens from pp32 Family Members. *Era of Hope* (BCRP): 3:54-2, 2002.

Conclusions:

We demonstrated *in vitro* that

(i) the oncogenic pp32 family members can be presented by HLA-A*0201,
(ii) the HLA-A*0201 cells bearing these motifs can be recognized and lyzed by pp32r1- or pp32r2- specific CTL in a MHC class I specific manner.

```
1                                                                    50
pp32     memgrrihle lrnrtpsdvk elvldnsrsn egklegltde feeleflsti
pp32r1           s      a                        a              k
pp32r2       kw                    f    q                    l n

         51                                                         100
pp32     nvgltsianl pklnklkkle lsdnrvsggl evlaekcpnl thlnlsgnki
pp32r1    g     sd     ~  r     ~~~k                    y
pp32r2    i                     s  a v              i

         101                                                        150
pp32     kdlstieplk klenlksldl fncevtnlnd yrenvfkllp qltyldgydr
pp32r1             q                      g       l       scyw
pp32r2                  e      t       n  ~~~~~~~~~ ~~~~~~~~~~

         151                                                        200
hpp32p   ddkeapdsda egyvegldde eededeeeyd edaqvvedee dedeeeegee
pp32r1    h    y  i  dh          g h                 g e
pp32r2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

         201                                                    249
hpp32p   edvsgeeeed eegyndgevd geedeeelge eergqkrkre pedegeddd
pp32r1        gd              g                  ~~ ~~~~~~~~~
pp32r2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
```

Figure 1. Alignment of pp32, pp32r1 & pp32r2 sequences.
Differences from the pp32 sequence are indicated underneath. The variant pp32r2 encodes a truncated protein (wavy lines indicate the truncated region).

Peptide	BIMAS	LpRep	FPEITHI	T2 Stabilization
0202-01	3499.535	3.37	26	+
0202-02	1591.602	2.46	22	-
0202-03	805.719	2.76	27	+
0202-04	681.542	3.54	18	+
0202-05	636.316	4.19	25	+
0202-06	481.542	6.90	27	-
0202-07	445.216	3.13	26	+
0202-08	432.319	4.87	21	-
0202-09	399.682	7.69	23	+
0202-10	379.216	5.81	13	-
0202-11	301.331	3.12	27	+
0202-12	281.542	3.47	22	-
0202-13	264.498	6.72	24	+
0202-14	226.014	3.54	20	-
0202-15	212.775	6.43	19	+
0202-16	172.752	6.81	21	+
0202-17	148.896	5.87	24	-
0202-18	139.730	6.72	19	-
0202-19	105.719	7.99	18	-
0202-20	103.362	6.79	21	-
MGA1	734.189	4.86	26	+

Table 1. Predicted HLA-A*0201 Binding Motifs and Their Ability to Bind T2 Cells.

Potential motifs was predicted by *BIMAS, LpRep, FPEITHI.*
The binding of Peptides to Human HLA-A2 was measured by T2 stabilization assay
Positive – calculated fluorescent index greater than 1.0.
Calculated fluorescent index = (Mean fluorescence with peptide - mean fluorescence without peptide)/(mean fluorescence without peptide)

Peptide	CTL Lysis*	Processing+	MHC I Restriction#
0202-01	+	n/a	n/a
0202-03	+++	Yes	Yes
0202-04	+	n/a	n/a
0202-05	+	n/a	n/a
0202-07	+++	Yes	Yes
0202-09	+	n/a	n/a
0202-11	-	n/a	n/a
0202-13	+	n/a	n/a
0202-15	-	n/a	n/a
0202-16	-	n/a	n/a
MGA1	+++	Yes	Yes
ID9	-	No	No

Table 2. Summary of CTL Assays for Motifs That are Capable of Binding to HLA-A*0201

Cytotoxicity Assay was carried out against Target cells:

* T2 Cell +/- peptides

\+ MCF-7 ($A2^+$, $pp32r1^+$, $pp32r2^+$)
LNCAP ($A2^+$, $pp32r1^-$, $pp32r2^-$)

\# MCF-7 (+/- anti-HLA-A2mAb)

Summary of Personnel Partially Supported by This Idea Award:

1) Jining Bai (PI)
2) Adetinuke Jagun/Tianzhi Mao (Technician)

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1996-1999	Post-doctoral Fellow, Division of Molecular Pathology Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD

Professional Experiences:

1985-1986	Instructor, Computer programming School of Professional Studies Tsinghua University, Beijing, P. R. China
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1988-1990	Graduate studies, Department of Biol. Sci. & Tech., Tsinghua University, Beijing, P. R. China
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1992-1993	Teaching Assistant, Reproductive Physiology Johns Hopkins University.
1996-2000	Research Fellow, Division of Molecular Pathology Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD
2000-2001	Research Associate, Division of Molecular Pathology Department of Pathology, Johns Hopkins Medical Institutions Baltimore, MD
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Bibliography:

Refereed Publications

Yu W, Jagun A., Zhu X, Jaffee EM and Bai, J. Identification of Candidate Tumor-Associated Antigens (TAA) from pp32 Family Members, *manuscript in preparation.*

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Absracts

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Bai, J. , Kadkol, S. S., Brody, J. R., Chamberlin, M., Cheong, R. & Pasternack, G.R. Cell- Type Specific Suppression of Proliferation of Human Prostatic Adenocarcinoma Cell by a Novel Tumor Suppressor pp32. *Proc. AACR.* Supl. 10:A-10, 1998

Invention & Patents:

Pasternack, G.R. & Bai, J. Method of Treating Cancer by Restoration of pp32 Function, *United States Patent & Trademark Office (USPTO)*, #60/118667, 1999.

Bai, J, & Yu W Aldehyde Dehydrogenase Isoform 8 for Therapy and Diagnosis of Breast Cancer, *in preparation.*

Bai, J.,Ibrahim G, & Yu W. Carboxypeptidases B for Therapy and Diagnosis of Breast Cancer, *in preparation.*

Bai, J, & Yu W Dermcidin for Therapy and Diagnosis of Breast Cancer, *in preparation.*

Grants & Contracts:

Current:

1) Idea Award — DOD/CDMRP — Principal Investigator
Active — (10/01-10/04) — $100,000 (annual direct)
Identification of Widely applicable Tumor- Associated Antigens for Breast Cancer ImmunoTherapy.

2) Pilot Award — Breast Cancer SPORE/oncology — Principal Investigator
Active — (09/02-09/03) — $40,000 (annual direct)
HOXB7, Widely Applicable Targets for Immunotherapy against Breast Cancer.

Honors & Awards:

Honored Student, Tsinghua University (1983-1988)
Outstanding College Graduate Award, National Education Commission of China (1988)
Winner of Natural Philosophy Competition, Tsinghua University (1990)
Travel Award, European Symposium in Signal Transduction (1991)
Carnegie Fellowship, Carnegie Institution of Washington (1990-1991)
Dean's Fellowship, Johns Hopkins University (1990-1995)
Pathology Fellowship, Johns Hopkins Medical Institution (1996-1999)
National Research Award, Susan G. Komen Breast Cancer Foundation (1999-2001)
Concept Award, Congressionally Directed Medical Research (2000-2001)
Idea Award, Congressionally Directed Medical Research (2001-2004)

Invited Lectures:

1) Alterations in pp32 Gene Family – A Novel Molecular Targets in Breast Cancer Therapy.
The 4th National Mission Conference for Breast Cancer
Washington D.C.
September, 2000

2) pp32 Gene Family, Potential Therapeutic Targets for Breast Cancer and Prostate Cancer .
National Cancer Institute

Beijing, P.R. China
October, 2000

3) pp32 Gene Family at the Crossroad of Oncogenesis and Tumor Suppression.
The Cancer Congress 2000
Beijing, P.R.China, October, 2000